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# *Pseudomonas fluorescens* pirates both ferrioxamine and ferri-coelichelin siderophores from *Streptomyces ambofaciens*

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Running Head: *P. fluorescens* pirates *Streptomyces* siderophores

**FOOTNOTES**

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**Keywords**

xenosiderophore, *Streptomyces ambofaciens*, *Pseudomonas fluorescens*, iron chelation, TonB-dependent receptor

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## Abstract

Iron is essential in many biological processes. However, its bioavailability is reduced in aerobic environments such as soil. To overcome this limitation, microorganisms have developed different strategies, such as iron chelation by siderophores. Some bacteria have even gained the ability to detect and utilize xenosiderophores, *i.e.* siderophores produced by other organisms. We illustrate an example of such an interaction between two soil bacteria, *Pseudomonas fluorescens* strain BBc6R8 and *Streptomyces ambofaciens* ATCC23877, which produce the siderophores pyoverdine and enantio-pyochelin and the siderophores desferrioxamines B, E and coelichelin, respectively. During pairwise cultures on iron-limiting agar medium, no induction of siderophore synthesis by *P. fluorescens* BBc6R8 was observed in presence of *S. ambofaciens* ATCC23877. Co-cultures with a *Streptomyces* mutant strain that produced either coelichelin or desferrioxamines, as well as culture in a medium supplemented with desferrioxamine B, resulted in the absence of pyoverdine production; however, culture with a double mutant deficient in desferrioxamines and coelichelin production did not. This strongly suggests that *P. fluorescens* BBbc6R8 utilizes the ferrioxamines and ferri-coelichelin produced by *S. ambofaciens* as xenosiderophores, and therefore no longer activates the production of its own siderophores. A screening of a library of *P. fluorescens* BBc6R8 mutants highlighted the involvement of the TonB-dependent receptor FoxA in this process: the expression of *fox*A and genes involved in the regulation of its biosynthesis was induced in presence of *S. ambofaciens*. In a competitive environment such as soil, siderophore piracy could well be one of the driving forces that determines the outcome of microbial competition.

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## Introduction

Bacteria detect, assimilate and integrate different environmental signals in order to better adapt to their habitat and to cope with changes in environmental conditions. Multiple signaling pathways allow them to communicate with each other within the same species or between different species (1). This can be achieved through the production and detection of diffusible molecules in the environment. In response to these interactions, the microorganisms have developed complex metabolic and physiological responses. One of the essential environmental factors vital for organisms is iron. It plays an essential role in many biological processes such as DNA synthesis, respiration and photosynthesis. Iron can adopt two different ionic forms $Fe^{2+}$ or $Fe^{3+}$. This property makes it an important player in the oxidation-reduction reactions in the cell. However, while iron is an abundant element on earth, its bioavailability is reduced in aerobic environments, as in soil. Ferric iron ($Fe^{3+}$) forms insoluble ferric hydroxides (solubility product of ~ $10^{-39}$) in the presence of oxygen (2–4). Therefore, iron is a limiting factor for the growth of microorganisms.

To overcome the limitation of iron bioavailability, aerobic bacteria have developed several highly specialized strategies to acquire this metal from different sources. One of them consists in producing siderophores under conditions of iron deficiency (5). Siderophores are low molecular weight molecules (200 to 2000 Da) with diverse iron affinities and to date, more than 500 different chemical structures of siderophores have been identified. A single bacterial species can produce different siderophores although in general, not all are produced at the same time. For example, *Pseudomonas aeruginosa* is able to switch between the synthesis of its siderophores, pyoverdine and pyochelin, depending on environmental conditions. In a severe iron depletion environment, pyoverdine, the most effective but metabolically expensive siderophore, is produced. However in environments moderately depleted

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in iron, pyochelin, a less metabolically expensive siderophore, is used to take up iron (6).

In addition to acquiring iron via specific receptors for their own siderophores, many bacteria possess uptake systems for xenosiderophores, *i.e.* siderophores produced by other organisms. Thus, in competitive environments like soil, this allows them to utilize exogenous siderophores, in a strategy known as siderophore piracy (7, 8). A study, published by Cornelis and Bodilis (2009), revealed that the majority of siderophore receptors are conserved in the different representatives of a species (the core receptor) while others are acquired by horizontal gene transfer. For example, *Pseudomonas fluorescens* SBW25 possess 23 other putative siderophore receptors in addition to the receptor of its own pyoverdine. This allows the bacterium to take up 19 heterologous pyoverdines from 25 different *Pseudomonas* isolates (9). *Pseudomonas fragi*, which does not produce siderophores, is able to use enterobactin, pyoverdine and desferrioxamine B produced by the bacterial species *E. coli*, *P. fluorescens* or *P. aeruginosa* and *Pseudomonas stutzeri*, respectively (10). Other genera of bacteria, like *Yersinia* (11), *Erwinia* (12), *Vibrio* (13), *Amycolatopsis* (14), are also able to detect and to take up xenosiderophores in addition to the use of their own siderophore. The wide distribution of xenosiderophore uptake genes in many bacteria species suggests that siderophore piracy is a common process in multispecies communities. However, most of these studies were performed *in vitro* using purified siderophores and little is known regarding the occurrence of siderophore piracy during biotic interactions.

Actinomycetes and Pseudomonads represent two of the major groups of bacteria found in soils and rhizospheres (15, 16) and are likely to simultaneously utilize similar ressources such as iron or even to compete for them. Both groups produce siderophores to take up this essential element but different types of molecules are

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105 produced by the two groups. Many Pseudomonads produce pyoverdines, a family of
106 high-affinity catecholate-hydroxamate siderophores while the actinomycetes of the
107 genus *Streptomyces*, for instance, secrete hydroxamate siderophores such as
108 desferrioxamines and coelichelin. In this study, we show, through a pairwise
109 interaction between the two soil bacteria *Streptomyces ambofaciens* ATCC23877
110 (17) and *Pseudomonas fluorescens* BBc6R8 (18, 19) that the *P. fluorescens* strain
111 uses the Streptomycete's siderophores and does not induce the production of its own
112 siderophores pyoverdine and enantio-pyochelin in the presence of *S. ambofaciens*
113 ATCC23877 when grown on iron limiting conditions. Our study reveals that
114 *P. fluorescens* recognizes *Streptomyces* siderophores through FoxA, a TonB-
115 dependent receptor, whose biosynthesis is induced in the presence of
116 *S. ambofaciens* ATCC23877 siderophores.
117

118 ## Materials and methods

119 **Bacterial strains, media and culture conditions**

120 All *Streptomyces*, *Pseudomonas* and *Escherichia coli* strains used in this work are
121 listed in Table 1. *Streptomyces* strains were manipulated as described by Kieser *et*
122 *al.* (20). *Pseudomonas* cell stocks were prepared by streaking each strain on
123 Trypticase soy agar (TSA) medium (containing kanamycin at 20 µg/ml for the
124 P18B10 and P28H6 mutants) and incubating plates for 48 hours at 26.5°C or at 37°C
125 for *P. aeruginosa* PA01. A single clone was resuspended in 50 µl water and spread
126 on TSA medium then grown for 48 hours. Cells were collected, centrifuged at 13,500
127 g for 2 minutes and the pellet was washed twice with sterile water. The pellet was
128 then resuspended with sterile water to an $OD_{600}$ 0.7 ($10^9$ Colony-Forming Units -CFU-
129 per ml).

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To analyse the effect of the *S. ambofaciens* ATCC23877 strain on the production of siderophores by *P. fluorescens* BBc6R8, a bioassay was set up on 26A agar medium (for 400 mL, Glucose: 0.4 g; Tryptone: 6 g; NaCl: 2 g; pH 7.2). *Streptomyces* and *Pseudomonas* were streaked side by side at 3 mm apart from each other and were incubated at 26.5°C (37°C with *P. aeruginosa* PA01) during 2 days. The streaks were made of 2 µl of a spore suspension of *S. ambofaciens* ATCC23877 at $10^9$ CFU/ml and 2 µl of *Pseudomonas* at $10^7$ CFU/ml. A control experiment with *Pseudomonas* alone was done for each incubation condition. When mentioned, the iron chelator 2, 2'-bipyridyl (200 µM), desferrioxamine B mesylate (200 µM) or $FeSO_4,7H_2O$ (12.5 µM) were added in 26A agar medium. For fluorescence detection, a Chemidoc XRS (Biorad) was used with UV transmission (302 nm).

To test the ability of the *S. coelicolor* Δ*des*Δ*cch* mutant (strain W13) to take up exogenous siderophores, two different experiments were carried out. The first experiment was as follows: 100 µl of a spore suspension of *S. coelicolor* M512 at $2x10^8$ CFU/ml or 100 µl of a cell suspension of *P. fluorescens* BBc6R8 at $2x10^7$ CFU/ml were spread on 26A agar plate supplemented or not with 2,2'-bipyridyl at 100 or 200 µM. After 3 days of growth at 26.5°C, plugs (8 mm of diameter) were obtained from confluent regions and then placed on 26A agar plates containing 200 µM of 2,2'-bipyridyl and evenly spread with spores of the W13 strain ($10^6$ or $10^9$ CFU). Plates were incubated at 26.5°C for 3 days. The halo of growth around the plugs was then analyzed. In the second experiment, the M512 and BBc6R8 strains ($10^4$ CFU) were streaked on both side of a 26A agar plate supplemented with 200 µM 2,2'-bipyridyl. Plates were incubated at 26.5°C for 3 days and the W13 mutant (2 µl at $10^9$ CFU/ml) was then streaked at about 3 mm alongside the M512 or BBc6R8 strains.

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156 **DNA manipulation and transcriptional analysis**

157 Isolation, cloning, and manipulation of DNA were carried out as previously described
158 for *Streptomyces* (21, 22), *Pseudomonas* (23) and *E. coli* (24). Amplification of DNA
159 fragments by PCR was performed with Dreamtaq DNA polymerase (Fermentas). All
160 primers are described in Table 2. Transposon insertion site in *P. fluorescens* BBc6R8
161 was determined using a double-round nested PCR-based sequencing approach.
162 First round PCR was performed using the primers gfptns2, nCEKG2A, nCEKG2B,
163 nCEKG2C with a ratio of 3:1:1:1. PCR conditions were as follows: 94ºC for 5
164 minutes, followed by cycles of 94ºC for 30 seconds, 62ºC for 30 seconds lowered in
165 successive cycles by 1ºC until reaching 54ºC and 72ºC for 3 minutes. A further 27
166 cycles annealing at 54ºC were performed and followed by a final extension of 72ºC
167 for 3 minutes. A second round PCR was performed using the primers gfptns2 and
168 CEKG4, followed by sequencing using the primer gfptnS2.

169 For RNA extraction, cells were collected from streaks grown on 26A agar medium for
170 2 days at 26.5°C. *Streptomyces* was grown on cellophane membranes. The
171 extraction was performed with Aurum™ Total RNA Mini kit (Biorad) according to the
172 manufacturer's instructions, except for the addition, during cell lysis, of a sonication
173 step (3x 10 s) at high frequency using the Bioruptor apparatus (Diagenode). RNAs
174 were eluted in a final volume of 30 µl and quantified with the NanoDrop-1000
175 apparatus. Before reverse transcription, RNA samples were treated with DnaseI (1 U
176 of Dnase I (Fermentas) in presence of 10 U of Ribolock Rnase Inhibitor (Fermentas)
177 per 1 µg of RNA). DNase I was then inactivated at 65°C during 10 min in presence
178 EDTA (2.3 mM). The absence of residual genomic DNA was verified by a 35 cycle
179 PCR using RNAs as templates and the primer pairs hrdB-F/hrdB-R and selR-F /selR-
180 R for *Streptomyces* and *Pseudomonas*, respectively. Reverse transcription was
181 performed with iScript Advanced cDNA synthesis kit for RT-qPCR (Biorad) according

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182 to the manufacturer's instructions. The sequences of primer pairs used to amplify
183 cDNAs and their target genes are listed in Table 2. Real-time quantitative PCR
184 (qPCR) were carried out on CFX96 (Biorad) with microplates (Multiplate™ 69-Well
185 Unskirted PCR plates, Low-Profil Biorad) covered with a film (Microseal'Bb'Adhesive
186 Seals, Biorad). The reaction mixture was composed of 5 µl of SYBR Green Supermix
187 (Biorad), 0.2 µM of each primer pairs and 4 µl of cDNA diluted 1/10. The qPCR
188 conditions were as follow: 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C.
189 To verify the absence of secondary products, melting curves were realized from 65 to
190 95°C with an increase of 0,5°C/cycle. Total RNA levels were normalized using
191 transcripts from the housekeeping genes *hrd*B (25) for *Streptomyces* and *sel*R
192 (MHB_002629, (26)) for *Pseudomonas* as a control. The gene *hrd*B encodes the
193 major sigma factor of *Streptomyces* and was used as an internal control to quantify
194 the relative expression of target genes as it is expressed fairly constantly throughout
195 growth (25, 27). *sel*R was defined, with two other genes, as a potential housekeeping
196 gene based on microarray data (accession number GSE38243 on the Gene
197 Expression Omnibus at NCBI, (26)) and was chosen as a reference after checking
198 the stability of its expression in the present setup. Both *hrd*B and *sel*R transcripts
199 levels were experimentaly confirmed to be stable in our growth conditions (data not
200 shown).
201 For each treatment, at least three biological replicates were performed and the
202 average and standard deviation of the relative expression to the reference genes
203 *hrd*B or *sel*R of each transcript were calculated (28). One factor ANOVA analyses
204 were made with R to test for differences of transcript levels between treatments.
205
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**Construction of *S. coelicolor* M512 *des*D and *cch*H mutant strains**

The cosmids C105.2.E01 and F76.2.F08 from the *S. coelicolor* transposon insertion single-gene knockout library (29) were used to replace the wild type alleles of *des*D and *cch*H, respectively, in *S. coelicolor* M512. Mutated cosmids were introduced in *S. coelicolor* M512 by intergenic conjugation from *E. coli* ET12567/pUZ8002 (30). Gene replacements were confirmed by PCR analysis using the flanking and the internal primers, desC-F and EZR1 for the mutant *S. coelicolor* M512 *des*D, sam0552-R and EZL2 for the mutant *S. coelicolor* M512 *cch*H.

## Results

***P. fluorescens* BBc6R8 does not produce its siderophore pyoverdine in the vicinity of *S. ambofaciens* ATCC23877**

*P. fluorescens* BBc6R8 produced a green-yellow pigment and fluoresced under UV when grown on iron depleted 26A agar medium (Fig. 1a, b). When the medium was supplemented with $FeSO_4,7H_2O$ (12.5 µM), *P. fluorescens* BBc6R8 did not fluoresce anymore (Fig. 1d). This green-yellow pigmentation reflects the presence of the pyoverdine siderophore produced by *P. fluorescens* BBc6R8 under conditions of iron limitation (31). During the screening of pairwise interactions between *S. ambofaciens* ATCC23877 and other soil bacteria, we observed that when grown on 26A agar medium in close proximity to *S. ambofaciens*, *P. fluorescens* BBc6R8 partially lost its ability to fluoresce (Fig. 1c, e, f). Similar results were observed in the presence of the high affinity iron chelator 2, 2'-bipyridyl (200 µM) in the agar medium (Fig. S1). Interestingly, a stronger effect was observed when *S. ambofaciens* ATCC23877 was cultivated on 26A agar for 30 hours before the Petri dish was inoculated with the BBc6R8 strain. Under this condition, neither the green-yellow pigment nor the fluorescence could be observed (Fig. 1g). Similar results were obtained when 2,2'-

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234 bipyridyl was present in the agar medium (Fig. S1). The negative effect on
235 pyoverdine production was proportional to the lag between the inoculation of the two
236 bacteria on the plate. In addition, streaking the bacterial partners perpendicularly to
237 one another way revealed that the effect of *S. ambofaciens* on the pyoverdine
238 production by *P. fluorescens* occurs only in the area surrounding the streak of
239 *Streptomyces* (Fig. 1h, i). Altogether, these data show that in the presence of *S.*
240 *ambofaciens* ATCC23877, the BBc6R8 strain does not produce its own siderophore.
241

242 **Transcription of pyoverdine and enantio-pyochelin synthesis genes is no**
243 **longer induced in the presence of *S. ambofaciens* ATCC23877**

244 The biosynthesis and uptake of pyoverdine as well as their regulation have been
245 thoroughly studied in *P. aeruginosa* (32–35) and in *P. fluorescens* SBW25 (9). A
246 search in the *P. fluorescens* BBc6R8 genome sequence (26) indicated that all the
247 genes involved in these processes are conserved in this strain (Table S1),
248 suggesting similar mechanisms of biosynthesis, uptake and regulation in the BBc6R8
249 strain as in *P. aeruginosa*. Therefore, we tested the influence of *S. ambofaciens* on
250 the expression of these genes by varying the duration of preincubation of
251 *Streptomyces* on the plate before the addition of *Pseudomonas*. Messenger RNAs
252 from cell lysates of *P. fluorescens* BBc6R8 grown on 26A agar medium in absence or
253 presence of *S. ambofaciens* were quantified using RT-qPCR. The duration of
254 preincubation of *Streptomyces* varied between 0 to 54h before the BBc6R8 strain
255 was streaked on the plate. The expression of genes involved in the biosynthesis of
256 pyoverdine (*pvd*D), its regulation (*pvd*O, *pvd*S, *pvd*Q) and export (*pvd*E), and in the
257 biosynthesis of the second type of siderophore of *P. fluorescens* BBc6R8, enantio-
258 pyochelin (*phc*F), were monitored after two days of growth. As expected, all
259 siderophore-related genes were transcribed when *Pseudomonas* was cultivated

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alone, reflecting the iron limitation in the 26A medium. In contrast, transcript levels of the pyoverdin genes were significantly reduced when *P. fluorescens* BBc6R8 was grown in the presence of *S. ambofaciens* (Fig. 2, $p<0.01$, one factor ANOVA) except for *pvd*D and *pvd*S when the two bacterial strains were simultaneously streaked on the agar plate. As for the production of pyoverdine, the decrease in transcript levels was proportional to the time lag between the seeding of the two bacterial strains on the plate for all genes analysed. The expression of the enantio-pyochelin *gene phc*F was similarly affected by the presence of *S. ambofaciens* ATCC23877. Altogether, these data confirm our initial observation that *P. fluorescens* BBc6R8 reduces and even stops producing its own siderophores on iron-limited medium in the presence of *S. ambofaciens* ATCC23877.

**_S. ambofaciens_ ATCC23877 expresses genes necessary for desferrioxamine and coelichelin production on 26A medium**

*S. ambofaciens* ATCC23877 produces two types of *tris*-hydroxamate siderophores: desferrioxamine (B and E) and coelichelin (36). We hypothesized that *S. ambofaciens* produces these siderophores when grown on 26A agar medium, and that these siderophores would then be accessible to the BBc6R8 strain. To test this hypothesis, we analysed the expression of the genes of the biosynthesis pathways of these two siderophores by RT-qPCR. The transcription of *des*C (acyl-CoA acyltransferase, desferrioxamine, (36)) and *sam*R0552/*cch*H (NRPS, coelichelin, (36)) was monitored over a two and half day time-course. Both genes were expressed under these conditions and their transcription peaked around 30 hours (Fig. 3). This indicates that *S. ambofaciens* also detects iron deficiency on 26A medium and induces the expression of its siderophore biosynthetic genes, strongly suggesting that desferrioxamine and coelichelin are produced.

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287 ***P. fluorescens* BBc6R8 utilizes siderophores produced by *Streptomyces***

288 Consequently, *P. fluorescens* BBc6R8 could use either ferrioxamine(s) and/or ferri-
289 coelichelin as xenosiderophores to cope with the lack of iron of the 26A medium. To
290 test this hypothesis, *P. fluorescens* BBc6R8 was cultivated on 26A agar plate
291 supplemented with desferrioxamine B mesylate at 200 µM. After 48 hours of culture,
292 *P. fluorescens* BBc6R8 did not fluoresce in presence of the exogenous purified
293 siderophore in contrast to the control (Fig. 4a, b). The addition of 8 µM of
294 desferrioxamine B in the medium was sufficient to inhibit pyoverdine production (Fig.
295 4c). This indicates that *P. fluorescens* BBc6R8 stops producing its own siderophore
296 in presence of desferrioxamine B, likely utilizing this compound as a
297 xenosiderophore.

298 *Streptomyces coelicolor* A3(2) produces the same siderophores as *S. ambofaciens*
299 ATCC23877 (36–38). Since cosmids mutated in the biosynthetic *des*D (type C
300 siderophore synthetase,(36)) and *cch*H (coelichelin NRPS, (38)) genes are available
301 from the *S. coelicolor* transposon insertion single-gene knockout library (29), we
302 decided to disrupt these genes in *S. coelicolor* M512 (a derivative of the *S. coelicolor*
303 A3(2) M145 strain unable to produce the pigmented antibiotics actinorhodin and
304 undecylprodigiosine (39)). Pairwise cultures between *P. fluorescens* BBc6R8 and
305 *S. coelicolor cch*H and *des*D mutants revealed that *Pseudomonas* did not fluoresce
306 in the presence of either of the two single mutants after 2 days of culture (Fig. 4d, e).
307 However, in the presence of the *S. coelicolor* Δ*des*Δ*cch* strain, a double mutant
308 deficient in the biosynthesis of desferrioxamine and coelichelin (36), the production of
309 the green-yellow pigment by *P. fluorescens* was not affected (Fig. 4f). Therefore, our
310 data suggest that *P. fluorescens* BBc6R8 has the ability to recognize and to take up

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both ferrioxamine and ferri-coelichelin and consequently no longer produces its own siderophores, pyoverdine and enantio-pyochelin.

**_Streptomyces_ is unable to take up _P. fluorescens_ BBc6R8 siderophores**

The question arose if *Streptomyces* conversely to *Pseudomonas* was able to use pyoverdin and/or enantio-pyochelin as xenosiderophores. It has been reported that the *S. coelicolor* Δ*des*Δ*cch* strain (W13 strain) cannot grow on agar medium supplemented with 200 µM 2, 2'-bipyridyl due to an extreme iron deficiency (36). Indeed, in our experimental setup, it was unable to grow on a 26A plate containing the iron chelator (data not shown). Therefore, we examined weither *P. fluorescens* BBc6R8 could compensate for this defiency by providing its own siderophores. A plug was collected from a 3 days culture of BBc6R8 on a 26A agar plate with or without 2, 2'-bipyridyl (200 µM) and placed onto a 26A agar plate supplemented with the iron chelator (200 µM) and evenly spread with the W13 strain. Alternatively, the W13 strain was streaked on a 26 A plate containing 2, 2'-bipyridyl (200 µM) alongside the BBc6R8 strain inoculated on plate 3 days earlier. As a control similar experiments were carried out with *S. coelicolor* M512 instead of *P. fluorescens* BBc6R8. While M512 efficiently promoted the growth of W13, BBc6R8 had no effect (Fig. S2). Therefore, we conclude that *S.coelicolor* is unable to use pyoverdine and enantio-pyochelin as xenosiderophores. By extension we expect a similar behavior for *S. ambofaciens* since the two species encode the same extracellular siderophore binding proteins ((40); data not shown).

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336 ***P. fluorescens* BBc6R8 uses a TonB-dependent receptor to detect**
337 ***S. ambofaciens* ATCC23877 ferri-siderophores**

338 To identify the genes of *P. fluorecens* BBc6R8 involved in the uptake of
339 ferrioxamines and ferri-coelichelin, pairwise cultures were performed on solid 26A
340 medium between *S. ambofaciens* ATCC23877 and 4.400 clones from a transposon
341 mutant library of *P. fluorescens* BBc6R8 (P. Burlinson and A. Deveau, pers. com.).
342 *S. ambofaciens* was streaked on plate 30 hours before the *Pseudomonas* mutants to
343 allow for accumulation of ferrioxamines and ferri-coelichelin in the medium. Two
344 mutants, P28H6 and P18B10, still fluoresced under UV in these conditions (Fig. 5a-
345 d). Similar results were observed when the P28H6 and P18B10 clones were grown
346 near the *S. coelicolor* M512 *des*D and *cch*H mutants unable to produce
347 desferrioxamine and coelichelin, respectively (Fig. 5e, f). In addition, production of
348 pyoverdine was also observed in the two *Pseudomonas* mutants when cultivated on
349 26A agar plate containing 200 µM of desferrioxamine B (Fig. 5g, h). Therefore, we
350 concluded that the mutants P28H6 and P18B10 of *P. fluorescens* BBc6R8 are no
351 longer able to recognize and/or to take up the *Streptomyces* siderophores. Analysis
352 of the mutants revealed that both P28H6 and P18B10 are mutated in the same gene,
353 the gene MHB_05767 (Fig. S3). A BLASTp search showed that the product of this
354 gene shares respectively 88%, 84% and 67% identity with FoxA, a TonB-dependent
355 ferrioxamine B receptor, of *P. fluorescens* Pf0-1, *Pseudomonas protegens* Pf-5 and
356 *P. aeruginosa* PAO1 (41, 42) (Fig. S4). By analogy, we named the gene of
357 *P. fluorescens* BBc6R8 *fox*A. From this set of experiments, we conclude that
358 *P. fluorescens* BBc6R8 would be able to detect and to take up the siderophores
359 ferrioxamine B and ferri-coelichelin via the outer membrane receptor FoxA.
360

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361 ***S. ambofaciens* ATCC23877 induces expression of genes involved in the TonB-**
362 **dependent receptor synthesis**

363 In *P. fluorescens* Pf0-1, *P. protegens* Pf-5 and *P. aeruginosa* PAO1, *fox*A belongs to
364 an operon with the genes *fox*I (encoding a ECF sigma factor), *fox*R (encoding an
365 anti-sigma factor) and *pep*SY (encoding a PepSY TM helix protein) which is under
366 the regulation of the Fur protein (42). Analysis of the genome sequence of *P.*
367 *fluorescens* BBc6R8 indicates that the homologs of genes *fox*I, *fox*R and *pep*SY
368 were also present in BBc6R8 (Table S1) and organized in a similar cluster as in *P.*
369 *fluorescens* Pf0-1, *P. protegens* Pf-5 and *P. aeruginosa* PAO1. A homolog of the *fur*
370 gene was also retrieved in the genome sequence of the BBc6R8 strain (Table S1).
371 This suggests that the mechanism of regulation of the desferrioxamine-mediated iron
372 uptake system in *P. fluorescens* BBc6R8 is similar to that of *P. aeruginosa* (32, 34).
373 After 24 h of incubation with *S. ambofaciens*, the expression level of *fox*A*, fox*I*, fox*R
374 *and pep*SY genes increased compared to the control treatment ($p<0.01$, one factor
375 (R8 alone) ANOVA, Fig 6). The transcription level of *fur* was significantly modified by
376 the presence of *S. ambofaciens* although the fold change was much smaller than for
377 the other transcripts (Fig 6, $p<0.01$ one factor (R8 alone) ANOVA). These data show
378 that in the presence of *S. ambofaciens*, and in conditions stimulating siderophore
379 production, the *P. fluorescens* BBc6R8 *fox*A transcriptional regulation cascade is
380 induced, most likely resulting in the production of the FoxA receptor and in the uptake
381 of the *Streptomyces* siderophores.

382

383 **Utilization of desferrioxamine is shared among other fluorescent**
384 **pseudomonads and *P. aeruginosa***

385 As similar biosynthetic and uptake systems are present in *P. fluorescens* BBc6R8,
386 *P. aeruginosa* PAO1 (41, 43), *P. protegens* Pf-5 and *P. fluorescens* Pf0-1 (42), we

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387 expected that these bacterial strains might also be able to react to the presence of
388 the siderophores produced by *S. ambofaciens* ATCC23877. Indeed, we found that *P.*
389 *fluorescens* Pf0-1, *P. aeruginosa* PAO1 and *P. protegens* Pf-5 no longer produced a
390 green pigment in presence of *S. ambofaciens* ATCC23877 and did not fluoresce
391 under UV. Interstingly, *P. fluorescens* SBW25, which does not have the *fox*A gene,
392 fluoresced even in presence of *S. ambofaciens* (Fig. S5). In the presence of purified
393 desferrioxamine B, *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1 and *P. protegens* Pf-5
394 no longer fluoresced, again in contrast to the strain SBW25 (Fig. S5). In co-cultures
395 with *S. coelicolor* M512 *desD*, *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1 and
396 *P. protegens* Pf-5 fluoresced less than in single culture, suggesting that they are also
397 able to utilize coelichelin (Fig. S5). Interestingly, a screening of a library of sixty
398 fluorescent pseudomonad strains isolated from forest soil (Table 1) revealed that all
399 these strains were also able to utilize *S. ambofaciens* siderophores (data not shown).
400

401 ## Discussion

402 Our study reports interspecies adaptative behaviour during common utilization of
403 limited iron ressources between the two soil inhabitants *Pseudomonas* and
404 *Streptomyces*. In particular, we show that, in iron deficient conditions, *P. fluorescens*
405 BBc6R8 does not induce the production of the fluorescent siderophore pyoverdine in
406 the presence of *S. ambofaciens* ATCC23877. Instead, *P. fluorescens* very likely
407 utilizes the *S. ambofaciens* siderophores desferrioxamine B and coelichelin as
408 xenosiderophores thanks to its FoxA receptor. In contrast to most studies in which
409 the use of xenosiderophores was revealed indirectly through the addition of purified
410 siderophore in the growth medium (44–47), binding affinity assays (10, 48), native
411 PAGE and surface plasmon resonance (49) or with labeled iron (10, 50, 51), we
412 revealed potential iron piracy through a direct interaction between a Gram-positive

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413 and a Gram-negative bacterium. This potential piracy likely occurs only in one
414 direction. Indeed, the growth of *S. coelicolor* Δ*des*Δ*cch* mutant under iron deficient
415 conditions could not be rescued in the presence of *P. fluorescens* BBc6R8 (Fig. S1).
416 Similar behaviour is expected from *S. ambofaciens* ATCC23877 since this strain is
417 extremely close phylogenetically to *S. coelicolor* and they encode the same
418 extracellular siderophore binding proteins. Similar piracy involving streptomycetes
419 has previously been reported but only with other actinomycetes (e.g. *Streptomyces*
420 and *Amycolatopsis* (14, 44, 52). It is interesting to note that these examples also
421 involved desferrioxamines, and that competition for iron could either stimulate (44,
422 52) or curtail (14) the growth and/or development of *Streptomyces*. The effect of the
423 siderophore piracy by *P. fluorescens* on *S. ambofaciens* remains to be identified.
424 Indeed, we could not observe any effect on the morphological differentiation of
425 *S. ambofaciens* as it only forms vegetative mycelium on the 26A medium.
426 Although our model bacteria were isolated from independent ecological niches
427 (*Laccaria bicolor* sporocarp for *P. fluorescens* BBc6R8 (53); soil in Picardie Region,
428 France, for *S. ambofaciens* (17)) and experiments were carried out in laboratory
429 growth conditions, this interaction for iron capture between *Streptomyces* and
430 *Pseudomonas* likely occurs in natural environments. Indeed, several studies have
431 reported that these bacterial genera share common ecological niches within soils
432 including environments such as the rhizosphere or bulk soils (54–56). Therefore, they
433 are expected to use common pools of scarce but essential elements such as iron.
434 The piracy would occur through the production by *P. fluorescens* BBc6R8 of the
435 TonB-dependent receptor FoxA. Interestingly, the BBc6R8 strain does not produce
436 ferrioxamine which is the most effective compound for iron scavenging, followed by
437 pyoverdine and enantio-pyochelin both of which it does produce (56). In a
438 competitive environment such as soil, it is certainly more advantageous to use

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439 efficient chelators produced by its neighbors rather than to produce its own
440 siderophores especially if they are less effective and metabolically costly as
441 demonstrated in (6). In addition, since desferrioxamines are synthesized by many soil
442 organisms, *P. fluorescens* BBc6R8 strain could obtain in this way iron at low
443 energetical cost in many competitive situations. Interestingly, it should be noted that
444 purified desferrioxamine added in the culture medium had a positive effect on the
445 growth of *P. fluorecens* BBc6R8 (data not shown). In contrast, *S. ambofaciens*
446 impacted negatively the growth of BBc6R8 in co-cultures, presumably because of the
447 secretion of secondary metabolites or/and through nutrient competition (data not
448 shown).

449 The production of desferrioxamines seems to be conserved throughout
450 Streptomycetes (57, 58). The *des*ABCD genes that direct the synthesis of these
451 chelating agents (37, 59) have been found in all *Streptomyces* sequenced genomes
452 and in other related genera, such as *Salinispora* (60). Furthermore, Kobayakawa and
453 coworkers detected by HPLC the production of desferrioxamines in 78% of their
454 *Streptomyces* collection (61). Desferrioxamines are also produced by other
455 actinomycetes and some Gram-negative bacteria (62, 63).

456 Our data indicate that the BBc6R8 outer membrane receptor FoxA would not only
457 allow the uptake of desferrrioxamines of *S. coelicolor* but also of the hydroxamate
458 siderophore ferri-coelichelin. The ability seems to be widespread amongst
459 fluorescent Pseudomonads. Indeed, the FoxA receptor is present in three different
460 subclades of the *P. fluorescens* genus (64) and based on our screening of sixty *P.*
461 *fluorescens* environmental strains, it is likely that they also possess a *fox*A gene and
462 even a *fox*A operon. A BLASTp analysis also showed that FoxA homologues are
463 present in other *Pseudomonas* species such as *P. stutzeri*, *P. aeruginosa*, *P. fulva*,
464 *P. putida* or *P. resinovorans* (data not shown) and also in different genera of bacteria

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465 like *Yersinia enterocolitica* (11) and *Erwinia Herbicola* (12). In these latter, FoxA is
466 known to bind a collection of ferrioxamine derivatives with different chain lengths or
467 bridges (65). The tris-hydroxamate siderophore coprogen is also recognized to a
468 certain extent by FoxA (65). Desferrioxamine B, coelichelin and coprogen are linear
469 siderophores (36, 65). These data suggest that FoxA could bind other linear ferric-
470 tris-hydroxamate siderophores (65). Therefore, *P. fluorescens* BBc6R8 may have the
471 ability to obtain a wider variety of siderophores than investigated in this study through
472 the production of the FoxA receptor and thus very efficiently compete with other
473 bacteria. FoxA could then be considered as an outer membrane receptor with a
474 broad spectrum, at least for siderophores of the tris-hydroxamate family, and as a
475 widespread receptor. Other bacteria, such as *Streptomyces* for example, also
476 possess broad-spectrum tris-hydroxamate receptors. Indeed, in *S. ambofaciens*
477 ATCC23877 and *S. coelicolor* A3(2), CdtB, a siderophore binding protein involved in
478 iron-siderophore transport, is able to bind ferrioxamines and ferri-coelichelin with high
479 affinity (36, 40). Moreover, DesE, a second siderophore binding protein binds
480 different ferric-tris-hydroxamates, with the exception of ferri-coelichelin (40). To
481 possess some broad spectrum siderophore receptors may be a frequent strategy
482 among microorganisms selected during evolution to be more competitive in soil.
483 The capability to take up xenosiderophores can be amplified if bacteria possess
484 multiple xenosiderophore receptors. This property is particularly well developed in the
485 genus *Pseudomonas*. Indeed, several *Pseudomonas* species possess numerous
486 TonB-dependent receptors, usually more than 20 with *P. protegens* Pf-5 encoding
487 the highest number (45) of TonB-dependent receptors (43). Twenty nine have been
488 identified so far in the draft genome sequence of the BBc6R8 strain (26). Several
489 bacteria possessing multiple siderophore binding proteins, like *Pseudomonas fragi*,
490 have even lost the ability to produce siderophores and rely exclusively on

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siderophore piracy (10). Whether siderophore piracy results from the loss of the ability to synthesize the cognate siderophores during evolution or the acquisition of receptor genes through horizontal transfer remains an open question. Nevertheless, our data and others strongly support the idea that the capacity for siderophore piracy is widespread among bacteria and that piracy by "cheaters" does happen *in vivo* (66). What is unclear is to which extent does this piracy really occur in natural ecosystems such as soils and how it impacts community dynamics. Metagenomic combined with metatranscriptomic may help answering this question in the future by providing a more complete picture of who is producing what and who is "cheating" in natural complex microbial communities.

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## Figure legends

**Fig. 1** Effect of *S. ambofaciens* ATCC23877 on green-yellow fluorescent pigment production by *P. fluorescens* BBc6R8. **a** and **b** *P. fluorescens* BBc6R8 (R8); **c** *S. ambofaciens* ATCC23877 (Sa); **d** *P. fluorescens* BBc6R8 cultivated on medium supplemented with $FeSO_4$; **e** and **f** *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877; **g** *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877 which has been streaked on plate 30 h before the BBc6R8 strain; **h** *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877 co-cultivated in a perpendicular way; **i** *P. fluorescens* BBc6R8 + *S. ambofaciens* ATCC23877 (streaked on plate 30 h before the BBc6R8 strain) co-cultivated in a perpendicular way. For parts b, c, d, f, g, h and i, plates were visualized under UV. Photographs were taken from below the plates after a two day (co-)cultureof incubation at 26.5°C on 26A agar medium.

**Fig. 2** Effect of *S. ambofaciens* ATCC23877 on the expression levels of *P. fluorescens* BBc6R8 genes involved in the biosynthesis of pyoverdine and enantio-pyochelin as measured by RT-qPCR. Expression levels are expressed as

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744 the transcript levels of target genes relative to the transcript level of the
745 housekeeping gene *sel*R which is stable in the tested growth conditions. Data are
746 expressed as the mean value of three biological replicates. Error bars denote
747 standard error. For each transcript, values with the same letter are not significantly
748 different according to a one-factor ANOVA (p>0.01). R8: *P. fluorescens* BBc6R8
749 grown on 26A agar medium in absence of *S. ambofaciens*. 0h to 54h: *P. fluorescens*
750 BBc6R8 grown in presence of *S. ambofaciens* ATCC23877 and *S. ambofaciens* was
751 streaked on 26A plate 0 h, 24 h, 30 h, 48 h or 54 h before the BBc6R8 strain. Total
752 RNAs were extracted 48 h after the plates were inoculated with *P. fluorescens* and
753 incubated at 26.5°C.
754
755 **Fig. 3** Expression levels of *S. ambofaciens* ATCC23877 genes controlling the
756 synthesis of desferrioxamine B and E and coelichelin as measured by RT-qPCR. The
757 *des*C gene (**A**) is involved in the synthesis of desferrioxamines and *sam*R0552 gene
758 (**B**) in the coelichelin biosynthesis. Total RNAs were extracted after 24, 30, 48 and 54
759 h of growth on 26A agar medium at 26.5°C. Expression levels are expressed as the
760 transcript levels of target genes relative to the transcript level of the housekeeping
761 gene *hrd*B which is stable in the tested growth conditions. Data are expressed as the
762 mean value of three biological replicates. Error bars denote standard error. For each
763 transcript, values with the same letter are not significantly different according to a
764 one-factor ANOVA (p>0.01).
765
766 **Fig. 4** Effect of desferrioxamine and coelichelin on the production of the pyoverdine
767 by *P. fluorescens* BBc6R8. **a** *P. fluorescens* BBc6R8 (R8); **b** *P. fluorescens* BBc6R8
768 cultivated in presence of 200 µM of desferrioxamine B mesylate; **c** *P. fluorescens*
769 BBc6R8 cultivated in presence of 8 µM of desferrioxamine B mesylate; **d** *P.*

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*fluorescens* BBc6R8 + *S. coelicolor* M512 *cch*H; **e** *P. fluorescens* BBc6R8 + *S. coelicolor* M512 *des*D; **f** *P. fluorescens* BBc6R8 + *S. coelicolor* Δ*des*Δ*cch*. The *Streptomyces* strains were streaked on plate 30 h before the inoculation of *P. fluorescens*. Photos were taken under UV from below the plates after a two day (co-)culture on 26A agar medium at 26.5°C.

**Fig. 5** Effect of the disruption of the *fox*A gene on the pyoverdine production by *P. fluorescens* BBc6R8. **a** *P. fluorescens* P28H6; **b** *P. fluorescens* P18B10; **c** *P. fluorescens* P28H6 or **d** *P. fluorescens* P18B10 cultivated with *S. ambofaciens* ATCC23877 (Sa); **e** *P. fluorescens* P28H6 cultivated with *S. coelicolor* M512 *cch*H and **f** with *S. coelicolor* M512 *des*D; **g** *P. fluorescens* P28H6 and **h** *P. fluorescens* P18B10 cultivated in presence of 200 µM of desferrioxamine B mesylate. The *Streptomyces* strains were streaked on plate 30 h before the inoculation of *P. fluorescens*. Photos were taken under UV from below the plates after a two day (co-)culture at 26.5°C on 26A agar medium.

**Fig. 6** Effect of *S. ambofaciens* ATCC23877 on the expression levels of *P. fluorescens* BBc6R8 genes controlling the synthesis of FoxA receptor. Analysis was done by RT-qPCR. R8 : *P. fluorescens* BBc6R8 grown on 26A agar in absence of *S. ambofaciens*. 0h to 54h: *P. fluorescens* BBc6R8 grown in presence of *S. ambofaciens* ATCC23877 which was streaked on solid 26A plate 0h, 24h, 30h, 48h or 54h before the BBc6R8 strain. Expression levels are expressed as the transcript levels of target genes relative to the transcript level of the housekeeping gene *sel*R which is stable in the tested growth conditions. Data are expressed as the mean value of three biological replicates. Error bars denote standard error. For each

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795 transcript, values with the same letter are not significantly different according to a
796 one-factor ANOVA (p>0.01).
797
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800
801 **Table 1** Bacterial strains used in this work

| Strains | Characteristics | References |
|---|---|---|
| *S. ambofaciens* ATCC23877 | Reference strain [wild type] | (17) |
| *S. coelicolor* M512 | Δ*red*D Δ*act*II-ORF4 from *S. coelicolor* M145; deficient in undecylprodigiosin and actinorhodin production | (39) |
| *S. coelicolor* M512 *des*D | Derived from M512, deficient in desferrioxamine production *S. coelicolor* M512 *des*D::Tn5062 | This study |
| *S. coelicolor* M512 *cch*H | Derived from M512, deficient in coelichelin production *S. coelicolor* M512 *cch*H::Tn5062 | This study |
| *S. coelicolor* Δ*des*Δ*cch* | Derived from M145, deficient in desferrioxamine and coelichelin production (*desEFABCD*:: *aac(3)IV*/*cchABCDEFGHIJK*::*vph*; strain W13) | (36) |
| *E. coli* ET12567(pUZ8002) | Donor strain in intergeneric conjugation, a methylation-defective strain | (30) |
| *P. fluorescens* BBc6R8 | Isolated from *Laccaria bicolor* | (18) |
| *P. fluorescens* SBW25 | Isolated from rhizosphere of sugar beet | (67) |
| *P. fluorescens* Pf0-1 | Isolated from agricultural soil | (68) |
| *P. aeruginosa* PAO1 | Human opportunistic pathogen | (69) |
| *P. fluorescens* P28H6 | Mutant derived from BBc6R8, Tn*5* insertion in *fox*A | (Deveau, unpublished) |
| *P. fluorescens* P18B10 | Mutant derived from BBc6R8, Tn*5* insertion in *fox*A | (Deveau, unpublished) |
| Library of 60 *P. fluorescens* strains | Strains isolated from the bulk soil of a forest nursery, the ectomycorrhizosphere and the ectomycorrhizas of *Laccaria bicolor*/Douglas fir | (70) |

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805 **Table 2** Primer pairs used in qPCR analyses or to determine transposon insertion sites in the

806 *P. fluorescens* BBc6R8 genome

| Name | Sequence 5'-3' | Studied gene |
|---|---|---|
| hrdB-F<br>hrdB-R | CGCGGCATGCTCTTCCT<br>AGGTGGCGTACGTGGAGAAC | *hrd*B |
| selR-F<br>selR-R | CGACCCGGAGCAGTACAA<br>GCAGCAGATGCAGTGGTAGA | *sel*R |
| pvdD-F<br>pvdD-R | TTGAGCGTGAGGACATTCTG<br>TCGAACCCTTGCAGGTAATC | *pvd*D |
| pvdE-F<br>pvdE-R | ACGTGGGGTGATCAATGAAT<br>TTTTGCCCCACATAATTGGT | *pvd*E |
| pvdO -F<br>pvdO –R | AAGAAAACCGGCCATCACTA<br>TGTCCACTCGTAGACGTTGC | *pvd*O |
| pvdS -F<br>pvdS –R | ACCATCACGTCATCGTTCAA<br>TTCTCCAGCGTCGAAAAGTT | *pvd*S |
| pvdQ-F<br>pvdQ-R | TGCGTTTCTACGAGATGCAC<br>AAATAGCGAGTCGGGTCCTT | *pvd*Q |
| phcf-F<br>phcf-R | ACGGCTACCAACAAATCCTG<br>CACCAGCAGATCCACTGAGA | *phc*F |
| desC-F<br>desC-R | ACTGACCGGGCTGTACGA<br>CTTCTCCGGCTTCTGGATCT | *des*C |
| sam0552-F<br>sam0552-R | CTTCGTCCTGCAGAACTTCC<br>AGTACGCGCAGGTAGTCGTC | *sam*0552 |
| foxI-F<br>foxI-R | ACGTGCGTTGATTCTGGAAT<br>GCACATAACAGTGATAAAGC | *fox*I |
| foxR-F<br>foxR-R | CGGCAGGCATTGAAGGTATT<br>CGCTATCGGTATTGAGCTGC | *fox*R |
| foxA-F<br>foxA-R | GCAGACAACGTGATCGAGAA<br>CACACCCTTCAGTCCAACCT | *fox*A |
| pepSY-F<br>pepSY-R | GTTTCTGATGATGGCGGGAC<br>ACTTGCAGTTTGGGATGCTG | *pep*SY |
| fur-F<br>fur-R | CCAGTTTGAAGCAGCAGGAC<br>TCCACCAAATCGAATCCATGC | *Fur* |
| EZR1 | ATGCGCTCCATCAAGAAGAG | Tn*5062-specific primer* |
| EZL2 | TCCAGCTCGACCAGGATG | Tn*5062-specific primer* |
| | | **Round of PCR** |
| gfptns2 | ATCACCTTCACCCTCTCCAC | For first and second round PCR of transposon mutants |
| nCEKG2A | ggccacgcgtcgactagtacnnnnnnnnnnagag | For first round PCR of transposon mutants |
| nCEKG2B | ggccacgcgtcgactagtacnnnnnnnnnnacgcc | For first round PCR of transposon mutants |
| nCEKG2C | ggccacgcgtcgactagtacnnnnnnnnnnatat | For first round PCR of transposon mutants |
| CEKG4 | GGCCACGCGTCGACTAGTAC | For second round PCR of transposon mutants |

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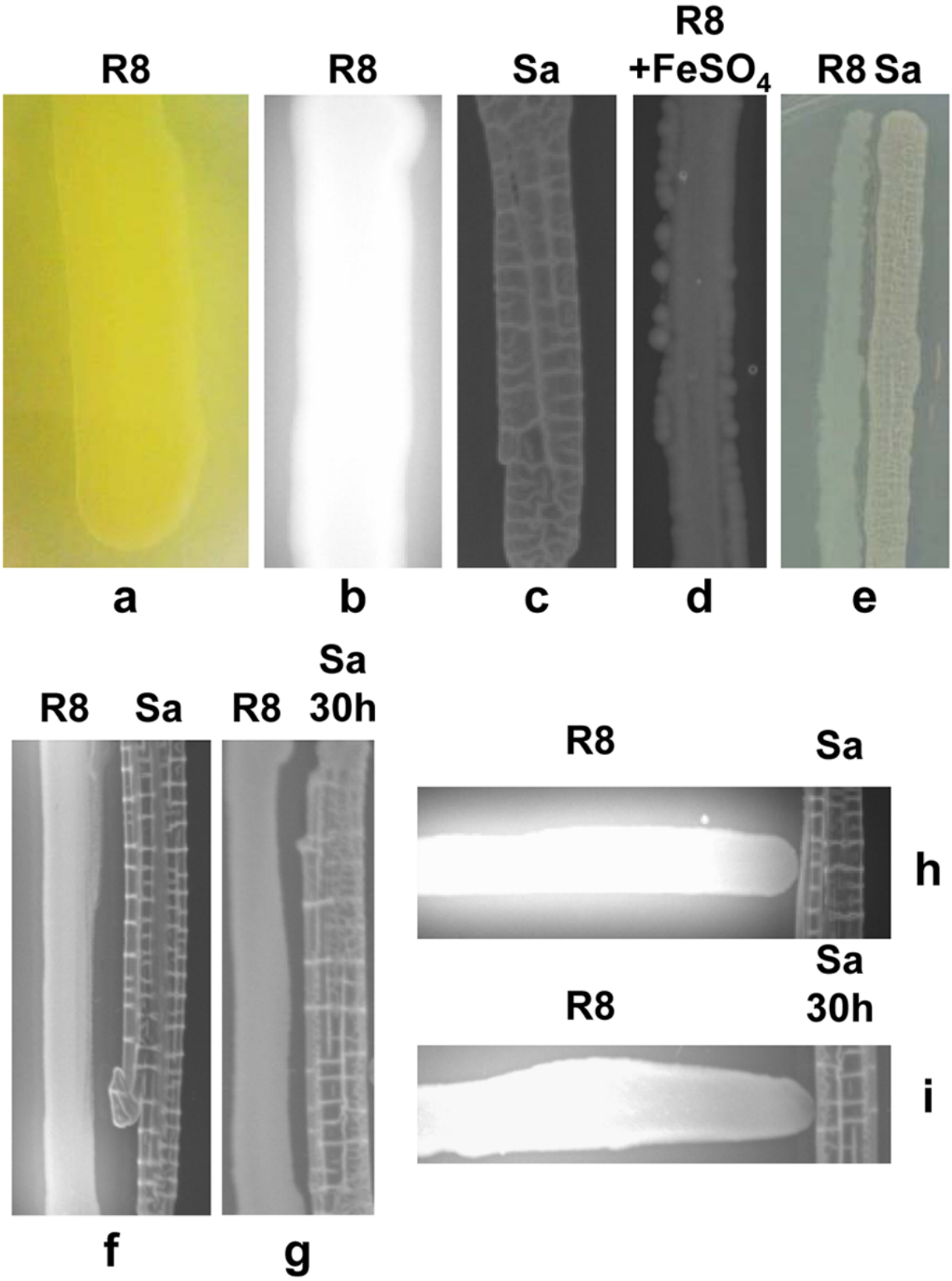


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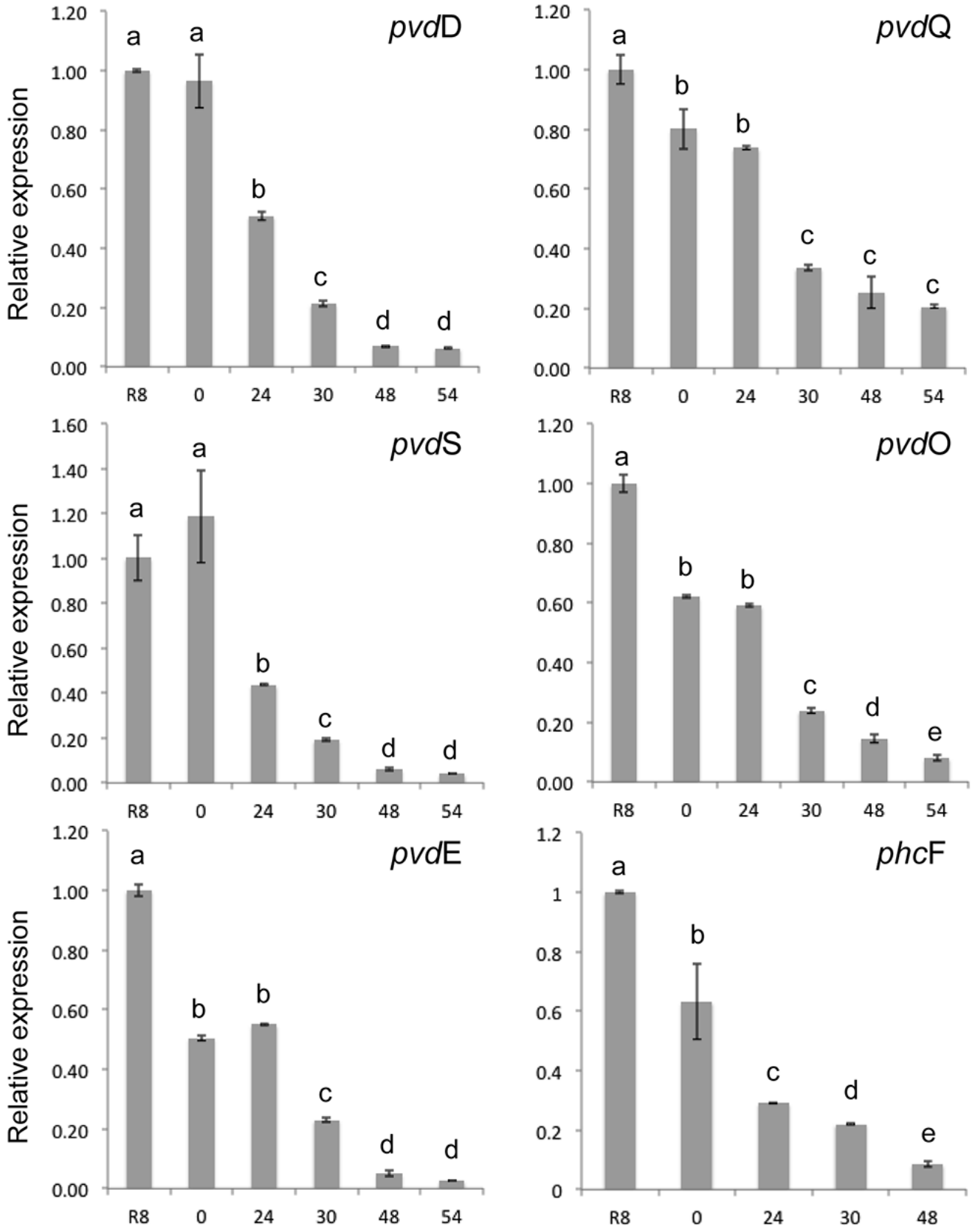


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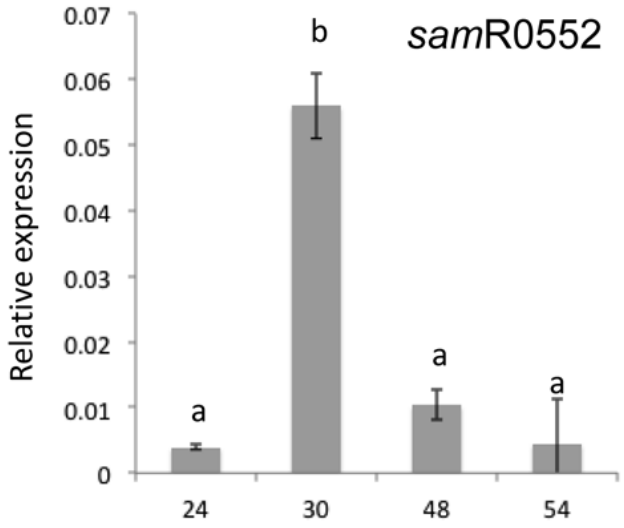


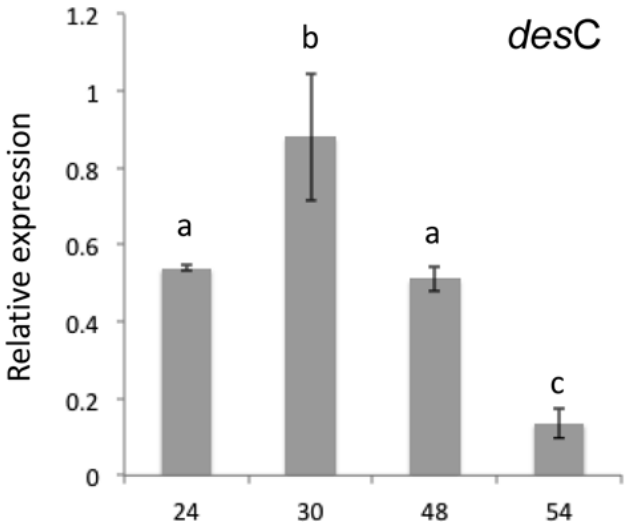


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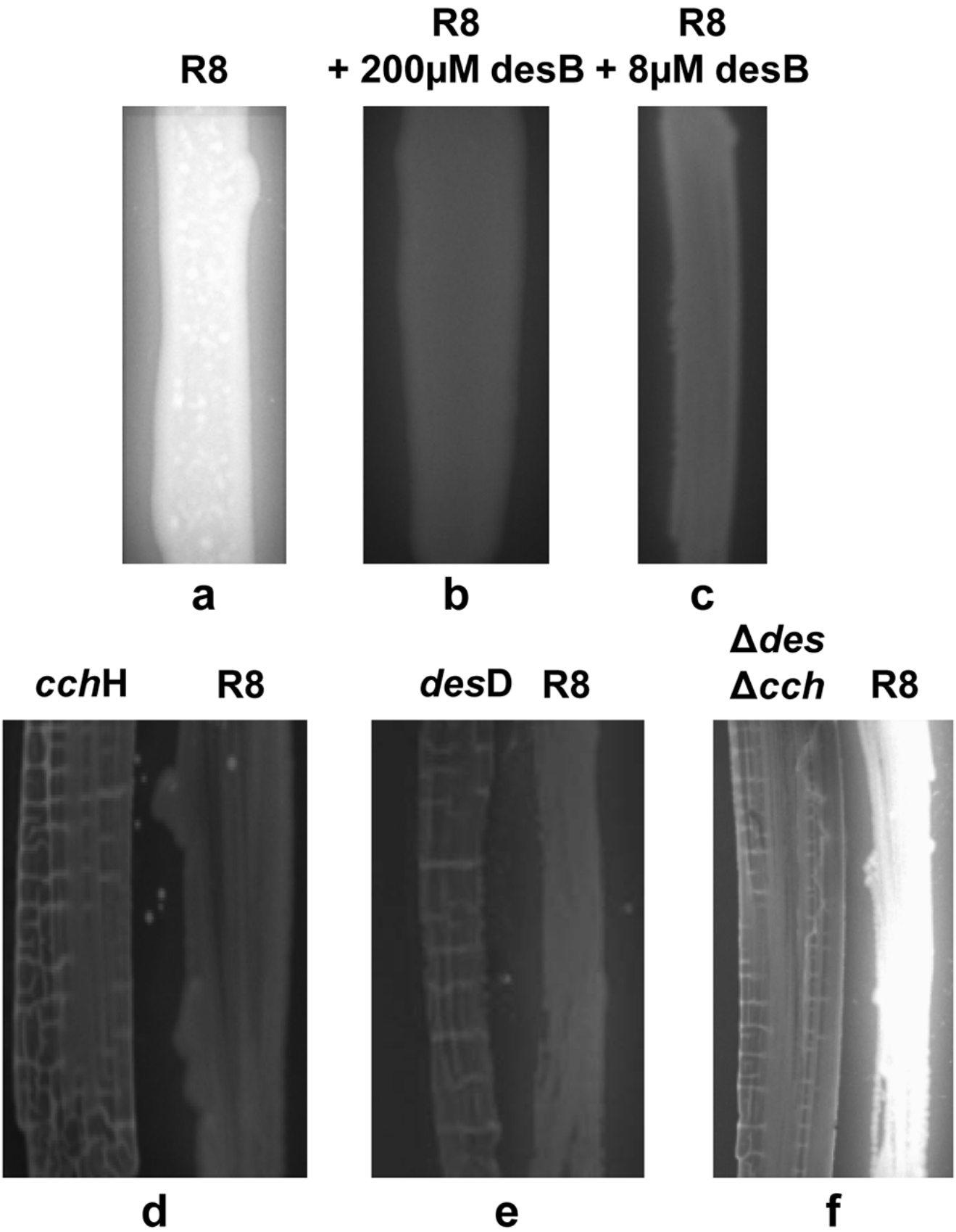


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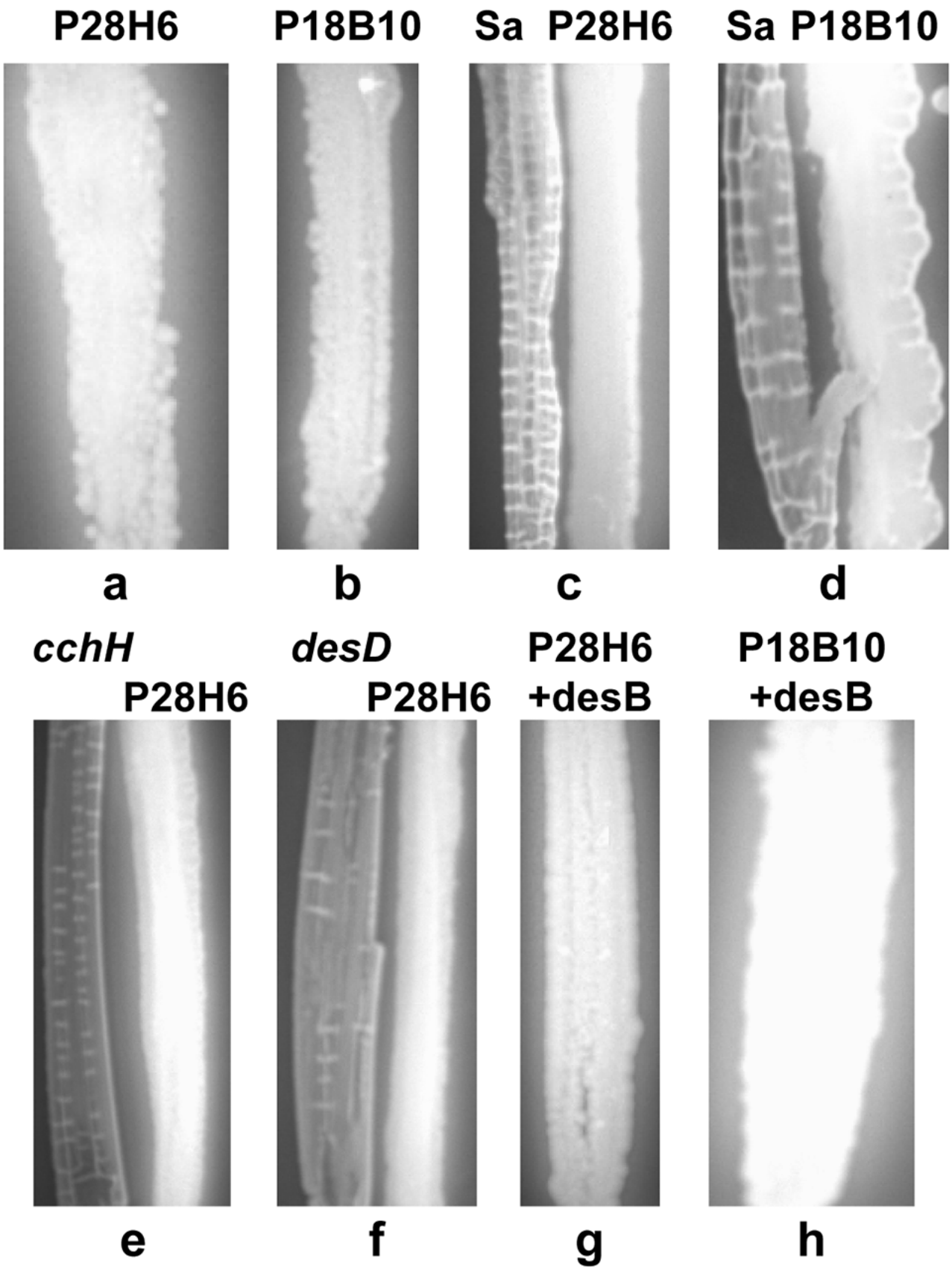


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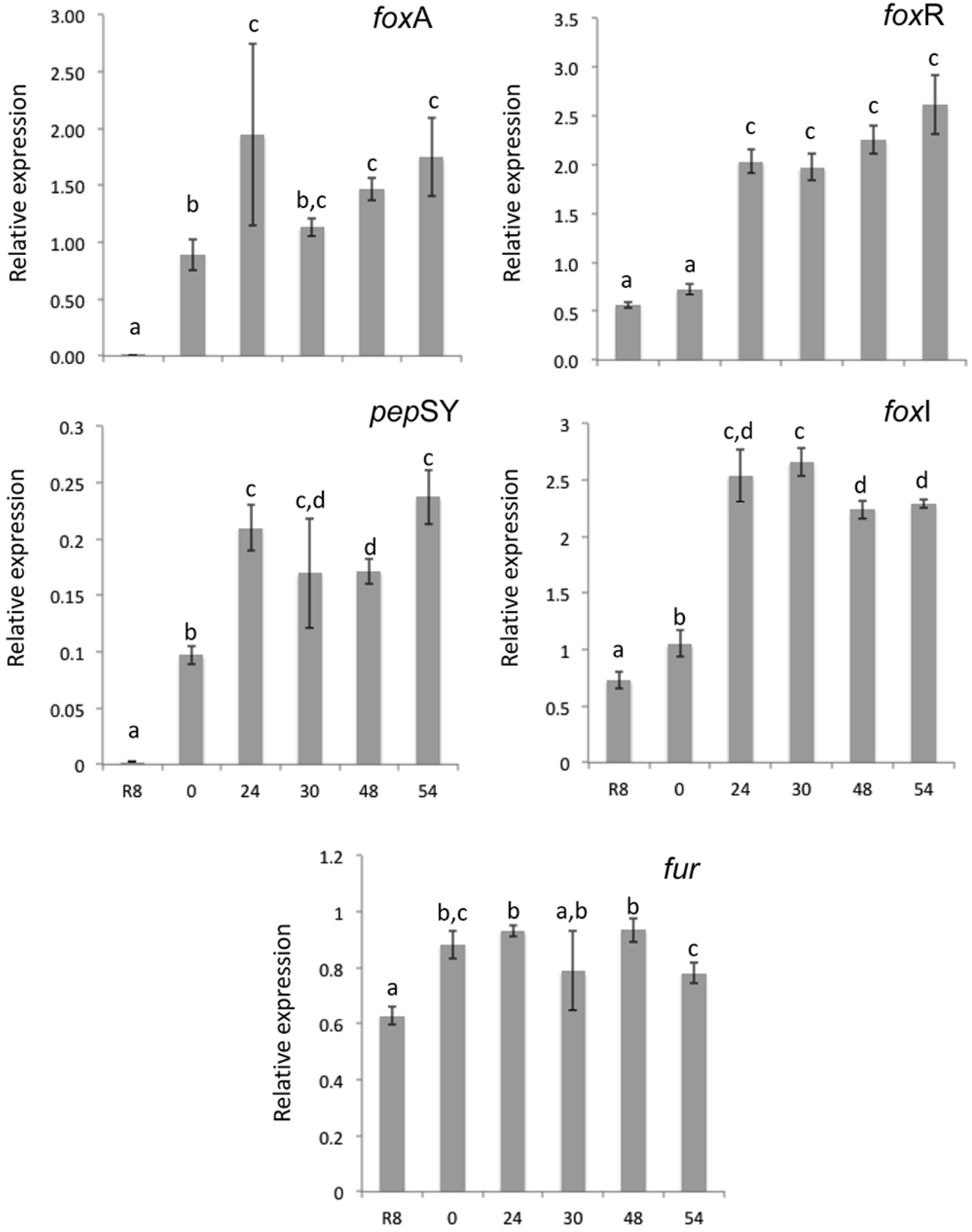


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